TITLE OF THE INVENTION

USE OF GABAPENTIN IN ASSAYS TO IDENTIFY $GABA_B$ RECEPTOR MODULATORS

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

The present invention is directed to novel methods of using gabapentin to identify agonists, inverse agonists, antagonists, and allosteric modulators of the $GABA_B$ receptor.

BACKGROUND OF THE INVENTION

GABA (γ-amino-butyric acid) is the most widely distributed amino acid inhibitory neurotransmitter in the vertebrate central nervous system. The biological activities of GABA are mediated by three types of GABA receptors: ionotropic $GABA_A$ receptors, metabotropic $GABA_B$ receptors, and ionotropic $GABA_C$ receptors.

$GABA_B$ receptors play a role in the mediation of late inhibitory postsynaptic potentials (IPSPs). $GABA_B$ receptors belong to the superfamily of seven transmembrane-spanning G-protein coupled receptors that are coupled to neuronal K^+ or Ca^{++} channels. $GABA_B$ receptor activation increases K^+ or decreases Ca^{++} conductance and also inhibits or potentiates stimulated adenylyl cyclase activity. The expression of $GABA_B$ receptors is widely distributed in the mammalian brain (*e.g.*, frontal cortex, cerebellar molecular layer, interpeduncular nucleus) and has been observed in many peripheral organs as well.

A large number of pharmacological activities have been attributed to $GABA_B$ receptor activation, *e.g.*, analgesia; hypothermia; catatonia; hypotension; reduction of memory consolidation and retention; and stimulation of insulin, growth hormone, and glucagon release (see Bowery, 1989, Trends Pharmacol. Sci. 10:401-407 for a review.) It is well accepted that $GABA_B$ receptor agonists and antagonists are pharmacologically useful. For example, the $GABA_B$ receptor agonist (-)baclofen, a structural analog of GABA, is a clinically effective muscle relaxant (Bowery & Pratt, 1992, Arzneim.-Forsch./Drug Res. 42:215-223). (-)Baclofen, as part of a racemic mixture with (+)baclofen, has been sold in the United States as a muscle relaxant under the name LIORESAL® since 1972.

Functional $GABA_B$ receptors result following the co-expression of two protein subunits having characteristics similar to those of the metabotropic glutamate receptors, *viz.*, a signal peptide sequence followed by a large N-terminal domain believed to represent a ligand binding pocket that precedes seven transmembrane spanning domains. The hallmark seven transmembrane spanning domains are typical of G-protein coupled receptors (GPCRs), although metabotropic glutamate receptors and $GABA_B$ receptor proteins are considerably larger than most GPCRs. Recombinant expression of the two $GABA_B$ receptor subunit proteins, either together or separately, demonstrated that functional $GABA_B$ receptors were formed only when both proteins were expressed in the same cell, most likely as heterodimers (Jones et al., 1998, Nature 396:674-679; White et al., 1998, Nature 396:679-682; Kaupmann et al., 1998, Nature 396:683-687; Kuner et al., 1999, Science 283:74-77; Ng et al., 1999, J. Biol. Chem. 274:7607-7610; and International Patent Application PCT/US99/02361, filed February 3, 1999). The $GABA_B$ receptor heterodimer is composed of a subunit known as $GABA_BR1a$ (or a splice variant known as $GABA_BR1b$) (Kaupmann et al., 1997, Nature 386:239-246) together with a subunit known variously as $GABA_BR2$ (White et al., 1998, Nature 396:679-682; and Jones et al., 1998, Nature 396:674-679), GBR2 (Kuner et al., 1999, Science 283:74-77), gb2 (Ng et al., 1999, J. Biol. Chem. 274;7607-7610; or HG20 (International Patent Application PCT/US99/02361, filed February 3, 1999). However, it remains possible that $GABA_B$ receptor monomers or homodimers are functional when in certain cellular environments.

The identification of the functional $GABA_B$ receptor heterodimer should permit the development of more effective drugs that target this receptor.

Current methods of drug discovery generally involve assessing the biological activity (*i.e.*, screening) of tens or hundreds of thousands of compounds in order to identify a small number of those compounds having a desired activity against a particular target. Where that target is the $GABA_B$ receptor, drug discovery requires assays that measure the ability of candidate compounds to modulate $GABA_B$ receptor activity where the assays are capable of high throughput. One possibility is to use cell-based assays where the cells express, perhaps through recombinant methods, both proteins that make up the functional $GABA_B$ receptor heterodimer.

Such assays will generally involve the use of a "positive" control, *i.e.*, an agonist, a compound that is known to positively regulate $GABA_B$ receptor activity. While such compounds are known to exist, their number is small and most are effective only at fairly high concentrations. It would be desirable to identify more, and more potent, agonists of the functional $GABA_B$ receptor for use as positive controls in assays to identify additional agonists and antagonists of the $GABA_B$ receptor.

Gabapentin is an anticonvulsant used for the treatment of refractory partial seizures and secondary generalized tonic-clonic seizures. It has been proposed to have mood-stabilizing properties and may be useful in neuropathies such as diabetic neuropathy or post-herpetic neuralgia. As many as 45% of patients with diabetes mellitus develop peripheral neuropathies. Gabapentin monotherapy appears to be efficacious for the treatment of pain and sleep interference associated with diabetic peripheral neuropathy and exhibits positive effects on mood and quality of life (Rowbotham. et al., 1998, J. Am. Med. Assn. 280:1837-1842). Post-herpetic neuralgia (PHN) is a syndrome of often intractable neuropathic pain following herpes zoster (shingles) that eludes effective treatment in many patients. Gabapentin is also effective in the treatment of pain and sleep interference associated with PHN. Mood and quality of life also improve with gabapentin therapy (Rowbotham et al., 1998, J. Am. Med. Assn. 280:1831-1836).

Gabpentin has been shown to be effective in reducing the number of partial seizures in patients with drug-resistant partial epilepsy (U.K. Gabapentin Study Group, 1990, Lancet, 335:1114-1117). Gabapentin has been studied for use in amyotrophic lateral sclerosis. It has been shown to have antihyperalgesic action in an inflammatory pain model (Field et al., 1997, Br. J. Pharmacol. 121:1519-1522).

Gabapentin's mechanism of action has been the object of much study, but no consensus has arisen. Various hypotheses have been proposed. For example, Taylor et al., 1998, Epilepsy Res. 29:233-249 list the following possibilities: (1) gabapentin crosses several membrane barriers in the body via a specific amino acid transporter (system L) and competes with leucine, isoleucine, valine, and phenylalanine for transport; (2) gabapentin increases the concentration and probably the rate of synthesis of GABA in the brain; (3) gabapentin binds with high affinity to a binding site in brain tissues that is associated with an auxiliary subunit of voltage-sensitive calcium channels; (4) gabapentin reduces the release of several monoamine neurotransmitters; (5) gabapentin inhibits voltage-activated sodium channels; (6) gabapentin increases serotonin concentrations in human whole blood, which may be relevant to neurobehavioral actions; and (7) gabapentin prevents neuronal death. See also Taylor, 1997, Rev. Neurol. (Paris) 153 (Suppl) 1:S39-S45 and Brown & Gee, 1998, J. Biol. Chem. 273:25458-25465 for other references discussing possible mechanisms of action for gabapentin.

It is noteworthy that gabapentin is believed not to act through $GABA_B$ receptors. See The Compendium of Pharmaceuticals and Specialties, Thirty-third edition,1988, pp. 1101-1102, Canadian Pharmacists Association, Ottawa, ON, CA, where it is stated that gabapentin "does not interact with GABA receptors." See also Rowbotham. et al., 1998, J. Am. Med. Assn. 280:1837-1842, at page 1838: "Its [*i.e.*, gabapentin's] mechanism of action has not yet been fully elucidated, but appears not to involve binding to GABA receptors [citing Goa & Sorkin, 1993, Drugs 46:409-427]." Field et al., 1997, Br. J. Pharmacol. 121:1513-1522 state: "Although gabapentin was originally designed as a GABA analogue which would penetrate into the CNS, it does not interact with either $GABA_A$ or $GABA_B$ receptors ..."

SUMMARY OF THE INVENTION

The present invention is based upon the discovery of an unexpected and novel mechanism of action for gabapentin: agonist activity at the functional $GABA_B$ receptor. The discovery of this mechanism of action allows for the development of assays for the identification of agonists and antagonists of the $GABA_B$ receptor that employ gabapentin. The use of gabapentin in such assays will permit the identification of compounds that have a similar spectrum of pharmacological activities as gabapentin, but that either are more potent, or have

fewer of the undesirable side effects of gabapentin. Such assays can also be used to identify antagonists of $GABA_B$ receptors. Such antagonists are expected to useful where it is desirable to diminish the activity of $GABA_B$ receptors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows that gabapentin modulates inwardly rectifying potassium channels in *Xenopus* oocytes that co-express HG20 and $GABA_BR1a$, resulting in a functional $GABA_B$ receptor.

Figure 2A-B shows the complete cDNA sequence of HG20 (SEQ.ID.NO.:1).

Figure 3 shows the complete amino acid sequence of HG20 (SEQ.ID.NO.:2).

Figure 4 shows the complete cDNA sequence of murine $GABA_BR1a$ (SEQ.ID.NO.:20). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 5 shows the complete amino acid sequence of murine $GABA_BR1a$ (SEQ.ID.NO.:21). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 6A-B shows the amino acid sequence (Figure 6A) (SEQ.ID.NO.:22) and nucleotide sequence (Figure 6B) (SEQ.ID.NO.:23) (GenBank accession number AJ012185) of a human $GABA_BR1a$.

Figure 7A-B shows the nucleotide sequence (SEQ.ID.NO.:24) (GenBank accession number Y11044) of a human $GABA_BR1a$.

Figure 8A-B shows the amino acid sequences of two proteins from *C. elegans* resembling mammalian $GABA_B$ receptors. Figure 8A shows the amino acid sequence of GB1 (SEQ.ID.NO.:27). Figure 8B shows the amino acid sequence of GB2 (SEQ.ID.NO.:28).

Figure 9A-E shows that gabapentin modulates inwardly rectifying potassium channels in *Xenopus* oocytes that co-express HG20 and $GABA_BR1a$, resulting in a functional $GABA_B$ receptor. Shown are results obtained with different batches of gabapentin (Figure 9A-C) in oocytes that co-express both HG20 and $GABA_BR1a$. Figure 9D-E shows the results of control experiments in which neither GABA nor gabapentin was able to activate inwardly rectifying potassium currents in oocytes that express only a single subunit of the $GABA_B$ receptor. In Figure 9D, the

oocytes expressed only the murine $GABA_BR1a$ subunit and not also HG20; in Figure 9E, the oocytes expressed only HG20 and not also the murine $GABA_BR1a$ subunit. The oocytes were exposed to 1 μM gabapentin or to 100 μM GABA.

Figure 10A-D shows that GABA, but not gabapentin or baclofen, is able to couple the activity of the $GABA_B$ receptor to changes in cAMP levels, as monitored by the *Xenopus* melanophore pigment aggregation assay. Figure 10A shows that GABA is able to cause pigment aggregation in *Xenopus* oocytes that co-express FLAG-HG20 and murine $GABA_BR1a$. Figure 10B shows that baclofen is unable to cause pigment aggregation in this system. Figure 10C shows that the response caused by GABA in this system is blocked by CGP71872, confirming that GABA is acting through the $GABA_B$ receptor. Figure 10D shows that CGP71872 has no effect on the results seen with baclofen. Figure 10E shows that the effect of GABA is not mediated by metabotropic glutamate receptor 4 or by dimers of metabotropic glutamate receptor 4 and either HG20 or $GABA_BR1a$. Figure 10F shows that gabapentin is unable to cause pigment aggregation in this system. HG208 is FLAG-HG20 (see Example 3); HG20N is full-length HG20.

Figure 11A-F shows that gabapentin, baclofen, GABA, and glutamic acid are capable of inhibiting the increase in cAMP concentration caused by treament of HEK293 cells with forskolin in HEK293 cells that have been transiently transfected with and that express heterodimers of HG20 and $GABA_BR1a$. FLAG-HG20 and murine $GABA_BR1a$ were transiently transfected into HEK293 cells that stably expressed β-lactamase under the control of a promoter that is driven by the cyclic AMP response element. The graphs plot the amount of blue versus green flourecence in the cells as analyzed in a FACSvantage cell analyzer. Points in the upper right quadrants represent cells having a relatively large amount of blue fluorescene, *i.e.*, cells in which β-lactamase has been activated by an increase in cAMP concentration. Figure 11A shows that only 0.44% of untreated cells appear in the upper right quadrant ("UR" in the tabulated values near the graphs). Figure 11B shows that when the cells are treated with forskolin alone (1 μM), 12.35% of the cells appear in the upper right quadrant, reflecting the increase in cAMP levels in these cells caused by forskolin. Figure 11C shows that co-treatment of the cells with GABA (100 μM) and forskolin resulted in a decreased amount (6.23% as opposed to 12.35%) of the cells appearing in the upper right quadrant. Co-treatment with 100 μM of baclofen (Figure 11D), gabapentin (Figure 11E) or glutamic acid (Figure 11F)

resulted in similar decreases of blue fluorescent cells as compared to treatment with forskolin alone.

Figure 12A-D shows the use of an assay employing the expression of β-lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor. FLAG-HG20 and murine $GABA_BR1a$ were transiently transfected into Chinese hamster ovary (CHO) cells that stably expressed β-lactamase under the control of an NFAT promoter and the promiscuous G-protein Gα16. 50,000 cells were analyzed by FACS after treatment with: Figure 12A, vehicle (control cells); Figure 12B, 100 μM GABA; Figure 12C, 100 μM baclofen; Figure 12D, 100 μM gabapentin. The cells in Figure 12B-D were more blue than the control cells (Figure 12A), confirming that gabapentin is an agonist of the $GABA_B$ receptor and that β-lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor can be used to identify agonists of the $GABA_B$ receptor.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this invention:

An HG20 polypeptide has "substantially the same biological activity" as native HG20 (*i.e.*, SEQ.ID.NO.:2) if that polypeptide has a K_d for a ligand that is no more than 5-fold greater than the K_d of native HG20 for the same ligand. An HG20 polypeptide also has "substantially the same biological activity" as HG20 if that polypeptide can form heterodimers with either a $GABA_BR1a$ or $GABA_BR1b$ polypeptide, thus forming a functional $GABA_B$ receptor.

"Functional $GABA_B$ receptor" refers to the receptor formed by co-expression of HG20 and $GABA_BR1a$ or $GABA_BR1b$, most likely resulting in a heterodimer of HG20 and either $GABA_BR1a$ or $GABA_BR1b$, where the functional $GABA_B$ receptor displays at least one functional response when exposed to $GABA_B$ receptor agonists such as GABA, gabapentin, or (-)baclofen. Examples of functional responses are: pigment aggregation in *Xenopus* melanophores, modulation of cAMP levels, coupling to inwardly rectifying potassium channels, mediation of late inhibitory postsynaptic potentials in neurons, increases in potassium conductance, and decreases in calcium conductance. One skilled in the art would be familiar with a variety of methods of measuring the functional responses of G-protein coupled

receptors such as the $GABA_B$ receptor (see, *e.g.*, Lerner, 1994, Trends Neurosci. 17:142-146 [changes in pigment distribution in melanophore cells]; Yokomizo et al., 1997, Nature 387:620-624 [changes in cAMP or calcium concentration; chemotaxis]; Howard et al., 1996, Science 273:974-977 [changes in membrane currents in *Xenopus* oocytes]; McKee et al., 1997, Mol. Endocrinol. 11:415-423 [changes in calcium concentration measured using the aequorin assay]; Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 [changes in inositol phosphate levels]). Depending upon the cells in which heterodimers of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ are expressed, and thus the G-proteins with which the functional $GABA_B$ receptor is coupled, certain of such methods may be appropriate for measuring the functional responses of such functional $GABA_B$ receptors. It is well with the competence of one skilled in the art to select the appropriate method of measuring functional responses for a given experimental system.

A $GABA_BR1a$ or $GABA_BR1b$ polypeptide has "substantially the same biological activity" as a native $GABA_BR1a$ or $GABA_BR1b$ polypeptide if that polypeptide has a K_d for an amino acid, amino acid analogue, $GABA_B$ receptor agonist, $GABA_B$ receptor antagonist (such as CGP71872), GABA, saclofen, (-)baclofen, or (L)-glutamic acid that is no more than 5-fold greater than the K_d of a native $GABA_BR1a$ or $GABA_BR1b$ polypeptide for the same amino acid, amino acid analogue, $GABA_B$ receptor agonist, $GABA_B$ receptor antagonist. A $GABA_BR1a$ or $GABA_BR1b$ polypeptide also has "substantially the same biological activity" as a native $GABA_BR1a$ or $GABA_BR1b$ polypeptide if that polypeptide can form heterodimers with an HG20 polypeptide, thus forming a functional $GABA_B$ receptor. Native $GABA_BR1a$ or $GABA_BR1b$ polypeptides include the murine $GABA_BR1a$ sequence shown as SEQ.ID.NO.:20; the rat $GABA_BR1a$ or $GABA_BR1b$ polypeptides disclosed in Kaupmann et al., 1997, Nature 386:239-246; the human $GABA_BR1a$ sequence disclosed in GenBank accession number AJ012185 (SEQ.ID.NO.:21); and the protein encoded by the DNA sequence disclosed in GenBank accession number Y11044 (SEQ.ID.NO.:23). Native $GABA_BR1a$ or $GABA_BR1b$ polypeptides also include the *C. elegans* GB1 and GB2 sequences (SEQ.ID.NO.:27 and SEQ.ID.NO.:28).

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue

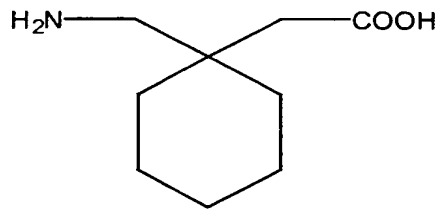
(isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (*e.g.*, arginine for lysine; glutamic acid for aspartic acid).

A "gabpentin-like compound" is a compound that is produced by modifying the structure of gabapentin by methods of medicinal chemistry. These modified gabapentin-like compounds may be, but are not necessarily, agonists or antagonists of the $GABA_B$ receptor. They can be tested by the assays described herein to determine if they are actually agonists or antagonists of the $GABA_B$ receptor.

A "gabapentin-like agonist" is a substance that is able to to couple the activity of the $GABA_B$ receptor to ion channels but is not able to couple the activity of the $GABA_B$ receptor to changes in melanophore pigment aggregation. Examples of gabapentin-like agonists are gabapentin and baclofen.

The present invention is based upon the discovery that gabapentin acts as an agonist at functional $GABA_B$ receptors resulting from the co-expression of HG20 and either $GABA_BR1a$ or $GABA_BR1b$. This discovery allows for the development of assays that can be used to identify compounds that are even more potent agonists of the $GABA_B$ receptor than gabapentin or that are antagonists of the $GABA_B$ receptor.

Gabapentin, 1-(aminomethyl)cyclohexaneacetic acid, has the following chemical structure:



According to the present invention, one can modify the structure of gabapentin by methods of medicinal chemistry well known in the art to produce compounds that are modified gabapentin-like structures. The modified structures can then be tested by methods disclosed herein to determine if they represent more potent agonists of the $GABA_B$ receptor than gabapentin or if they represent antagonists of the $GABA_B$ receptor. These compounds having modified gabapentin-like structures should be useful in the treatment of normal and diseased processes associated with

$GABA_B$ receptor expressing tissues. For example, in the brain, uses for gabapentin-like structures at $GABA_B$ receptors would be as neuroprotectants, cognitive enhancers, or antiapoptotic agents. In the periphery, uses for gabapentin-like structures would include use in pulmonary (asthma), urinary (incontinence), and intestinal disorders and in neuropathies.

The present inventors have discoved that gabapentin modulates inwardly rectifying potassium channels in *Xenopus* oocytes that co-express HG20 and $GABA_BR1a$ (resulting in the formation of a functional $GABA_B$ receptor). Oocytes were prepared and injected with RNA encoding HG20, $GABA_BR1a$, and human Kir 3.1 and Kir 3.2 potassium channels, without the G-protein subunit Giα1. The injected oocytes were exposed to gabapentin, GABA, and/or the $GABA_B$ receptor-specific antagonist CGP71872 (Kaupmann et al., 1997, Nature 386:239-246) and currents were recorded. See Example 4 for details. See Examples 1-3 for details of the HG20 and $GABA_BR1a$ constructs used.

Figure 1 shows the results. When the oocytes were treated with 1μ M gabapentin (Figure 1A), an inwardly rectifying potassium current was observed. Following washout, the membrane potential returned to resting state. The subsequent adddition of 100 μM GABA also resulted in the generation of an inwardly rectifying potassium current that was abolished by washout. Figure 1B shows that the $GABA_B$ receptor-specific antagonist CGP71872 prevented the generation of an inwardly rectifying potassium current by gabapentin, demonstrating that the current arose through action by gabapentin at the $GABA_B$ receptor. These data show that gabapentin acts as an agonist at the functional $GABA_B$ receptor formed by co-expression of HG20 and $GABA_BR1a$.

Figure 9A-C also shows that gabapentin modulates inwardly rectifying potassium channels in *Xenopus* oocytes that co-express HG20 and $GABA_BR1a$, resulting in the formation of a functional $GABA_B$ receptor. Oocytes were prepared and injected with RNA encoding HG20, murine $GABA_BR1a$, and human Kir 3.1 and Kir 3.2 potassium channels, without the G-protein subunit Giα1. The injected oocytes were exposed to gabapentin and GABA as indicated in Figure 9A-C and currents were recorded. Experimental details were the same as those in Examples 1-4.

Figure 9D-E shows that results of control experiments which demonstrate that both HG20 and $GABA_BR1a$ must be present in order for either

GABA or gabapentin to activate potassium currents. In Figure 9D, the experiment was as described above except that the oocytes were injected with RNA encoding murine $GABA_BR1a$ but were not also injected with RNA encoding HG20; in Figure 9E, the experiment was as described above except that the oocytes were injected with RNA encoding HG20 but were not also injected with RNA encoding $GABA_BR1a$. In both cases where a $GABA_B$ receptor subunit was lacking, inwardly rectifying potassium currents were not seen.

The present invention employs cells co-expressing HG20 and $GABA_BR1a$ or $GABA_BR1b$, resulting in the formation of functional $GABA_B$ receptors and HG20/$GABA_BR1a$ or $GABA_BR1b$ heterodimers. Such cells are generally produced by transfecting cells that do not normally express functional $GABA_B$ receptors with expression vectors encoding HG20 and $GABA_BR1a$ or $GABA_BR1b$ and then culturing the cells under conditions such that functional $GABA_B$ receptors and heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed where the heterodimers constitute functional $GABA_B$ receptors. The present invention may also employ cell lines derived from cerebellum or cortex which naturally express functional $GABA_B$ receptors. In this way, recombinant host cells expressing $GABA_B$ receptors are produced. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cells and cell lines which are suitable for recombinant expression of HG20 and $GABA_BR1a$ or $GABA_BR1b$ and which are commercially available, include but are not limited to, L cells L-M(TK^-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes.

Cells that are particularly suitable for use in the present invention are melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays that employ recombinant expression of HG20 and $GABA_BR1a$ or $GABA_BR1b$ in a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant GPCRs (Graminski et al.,

1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al., 1992, Anal. Biochem. 206:315-322). Especially preferred are *Xenopus* melanophore pigment cells co-expressing HG20 and $GABA_BR1a$ or $GABA_BR1b$, in which HG20 has formed a heterodimer with $GABA_BR1a$ or $GABA_BR1b$, thus forming a functional $GABA_B$ receptor. The presence of functional $GABA_B$ receptors in such cells can be determined by the use of assays such as the pigment aggregation assay described in the references cited above. Other assays that reflect a decrease in cAMP levels mediated by exposure to GABA or other agonists of $GABA_B$ receptors would also be suitable.

Other suitable cells are stably or transiently transfected HEK293 cells co-expressing HG20 and $GABA_BR1a$ or $GABA_BR1b$, in which HG20 has formed a heterodimer with $GABA_BR1a$ or $GABA_BR1b$, thus forming a functional $GABA_B$ receptor. The presence of functional $GABA_B$ receptors in such cells can be determined by the use of assays such as those that measure changes in cAMP levels.

Also suitable are *Xenopus* oocytes co-expressing HG20 and $GABA_BR1a$ or $GABA_BR1b$, in which HG20 has formed a heterodimer with $GABA_BR1a$ or $GABA_BR1b$, thus forming a functional $GABA_B$ receptor. The presence of functional $GABA_B$ receptors in such cells can be determined by the use of assays that measure coupling of functional $GABA_B$ receptors comprising heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ to inwardly rectifying potassium channels (especially the Kir3 family). In the case of *Xenopus* oocytes, co-expression of HG20 and $GABA_BR1a$ or $GABA_BR1b$ is often effected by microinjecting RNA encoding HG20 and RNA encoding $GABA_BR1a$ or $GABA_BR1b$ into the oocytes rather than by transfecting the oocytes with expression vectors encoding HG20 and $GABA_BR1a$ or $GABA_BR1b$. Microinjection of RNA into *Xenopus* oocytes in order to express proteins encoded by the RNA is well known in the art.

Also suitable are cells that express polypeptides that comprise amino acid sequences that are similar to, but not exactly the same, as the amino acid sequences disclosed herein for HG20, $GABA_BR1a$, and $GABA_BR1b$. It is generally accepted that single amino acid substitutions do not usually alter the biological activity of a protein (see, *e.g.*, Molecular Biology of the Gene, Watson et al., 1987, Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and

Cunningham & Wells, 1989, Science 244:1081-1085). Accordingly, suitable cells for the practice of the present invention include cells containing polypeptides where one amino acid substitution has been made in the HG20, GABA$_B$R1a, and GABA$_B$R1b amino acid sequences disclosed herein where the polypeptides still retain substantially the same biological activity as native HG20, GABA$_B$R1a, and GABA$_B$R1b. The present invention also includes polypeptides where two or more amino acid substitutions have been made in HG20, GABA$_B$R1a, and GABA$_B$R1b amino acid sequences disclosed herein where the polypeptides still retain substantially the same biological activity as native HG20, GABA$_B$R1a, and GABA$_B$R1b. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described substitutions do not occur in the ligand-binding domain of HG20, GABA$_B$R1a, and GABA$_B$R1b. In particular, the present invention includes embodiments where amino acid changes have been made in positions of HG20, GABA$_B$R1a, and GABA$_B$R1b that have not been evolutionarily conserved. For guidance as to which positions of HG20, GABA$_B$R1a, and GABA$_B$R1b have not been evolutionarily conserved, one of skill in the art can turn to disclosures such as Figure 1A of Kuner et al., 1999, Science 283:74-77; Figure Figure 1a of Kaupmann et al., 1998, Nature 396:683-687; Figure 1a of Jones et al., 1998, Nature 396:674-679; or Figure 1 of White et al., 1998, Nature 396:679-682. Such figures compare the amino acid sequence of HG20 with the amino acid sequences of GABA$_B$R1a or GABA$_B$R1b. Positions in which HG20 does not share the same amino acid as GABA$_B$R1a or GABA$_B$R1b are positions that have not been evolutionarily conserved.

In order to produce the above-described cells co-expressing HG20 and GABA$_B$R1a or GABA$_B$R1b, expression vectors comprising DNA encoding HG20 and GABA$_B$R1a or GABA$_B$R1b can be transfected into the cells. HG20 and GABA$_B$R1a or GABA$_B$R1b can be transfected separately, each on its own expression vector, or, alternatively, a single expression vector encoding both HG20 and GABA$_B$R1a or GABA$_B$R1b can be used.

A variety of expression vectors can be used to express recombinant HG20, GABA$_B$R1a, or GABA$_B$R1b. Commercially available expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen),

EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). The choice of vector will depend upon cell type used, level of expression desired, and the like.

The present invention provides methods of identifying compounds that specifically bind to the $GABA_B$ receptor. The specificity of binding of compounds having affinity for the $GABA_B$ receptor is shown by measuring the affinity of the compounds for recombinant cells expressing HG20 and either $GABA_BR1a$ or $GABA_BR1b$, or for membranes from such cells, in the presence of gabapentin. Expression of the $GABA_B$ receptor and screening for compounds that inhibit the binding of gabapentin to the $GABA_B$ receptor in these cells, or membranes prepared from these cells, provides an effective method for the rapid selection of compounds with high affinity for the $GABA_B$ receptor. Compounds identified by the methods disclosed herein are likely to be agonists or antagonists of the $GABA_B$ receptor and may be peptides, proteins, or non-proteinaceous organic molecules.

Accordingly, the present invention includes a method for determining whether a substance binds $GABA_B$ receptors and is thus a potential agonist or antagonist of the $GABA_B$ receptor that comprises:

(a) providing cells comprising an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$;

(b) culturing the cells under conditions such that HG20 and $GABA_BR1a$ or $GABA_BR1b$ are expressed and heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ are formed;

(c) exposing the cells to gabapentin in the presence and in the absence of the substance;

(d) measuring the binding of gabapentin to the heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ in the presence and in the absence of the substance;

where if the amount of binding of gabapentin is less in the presence of the substance than in the absence of the substance, then the substance binds $GABA_B$ receptors and thus is a potential agonist or antagonist of $GABA_B$ receptors.

Optionally, control experiments can be run utilizing control cells that are the same as the cells of step (a) except that the control cells do not comprise an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$. Preferably, the control cells do not express functional $GABA_B$ receptors. The amount of binding of gabapentin to the control cells should be significantly less than the amount of binding of gabapentin to the cells of step (a). In this way, one can ensure that the binding of gabapentin in step (d) is actually due to binding of gabapentin to $GABA_B$ receptors. Therefore, if the substance can block this binding, the substance is also likely to bind to $GABA_B$ receptors.

The present invention includes assays by which $GABA_B$ receptor antagonists may be identified by their ability to antagonize a functional response mediated by the $GABA_B$ receptor in cells that have been co-transfected with and that co-express HG20 and $GABA_BR1a$ or $GABA_BR1b$ and that have been exposed to gabapentin. Such a method comprises:

(a) providing cells comprising an expression vector encoding HG20 and an expression vector encoding $GABA_BR1a$ or $GABA_BR1b$;

(b) exposing the cells to gabapentin;

(c) measuring the amount of a functional response of the cells that have been exposed to gabapentin;

(d) exposing the cells concurrently to gabapentin and to a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(e) measuring the amount of the functional response of the cells that have been exposed to the substance and to gabapentin;

(f) comparing the amount of the functional response measured in step (c) with the amount of the functional response measured in step (e);

where if the amount of the functional response measured in step (c) is greater than the amount of the functional response measured in step (e), the substance is an antagonist of the $GABA_B$ receptor.

In particular embodiments, the functional response is selected from the group consisting of: modulation of the activity of an ion channel; changes in calcium concentration; changes in a signal from a reporter gene whose expression is controlled by a promoter that is induced by interaction of an agonist with the $GABA_B$ receptor; and changes in membrane currents. In particular embodiments, the change in membrane current is measured in *Xenopus* oocytes. In other embodiments, the

change in membrane current is the modulation of an inwardly rectifying potassium current.

Particular types of functional assays that can be used to identify agonists and antagonists of $GABA_B$ receptors include transcription-based assays. Transcription-based assays involve the use of a reporter gene whose transcription is driven by an inducible promoter whose activity is regulated by a particular intracellular event such as, *e.g.*, changes in intracellular calcium levels that are caused by the interaction of a receptor with a ligand. Transciption-based assays are reviewed in Rutter et al., 1998, Chemistry & Biology 5:R285-R290.

The transcription-based assays of the present invention rely on the expression of reporter genes whose transcription is activated or repressed as a result of intracellular events that are caused by the interaction of an agonist such as gabapentin with a heterodimer of HG20 and either $GABA_BR1a$ or $GABA_BR1b$ where the heterodimer forms a functional $GABA_B$ receptor.

An extremely sensitive transcription based assay is disclosed in Zlokarnik et al., 1998, Science 279:84-88 (Zlokarnik) and also in U.S. Patent No. 5,741,657. The assay disclosed in Zlokarnik and U.S. Patent No. 5,741,657 employs a plasmid encoding β-lactamase under the control of an inducible promoter. This plasmid is transfected into cells together with a plasmid encoding a receptor for which it is desired to identify agonists. The inducible promoter on the β-lactamase is chosen so that it responds to at least one intracellular signal that is generated when an agonist binds to the receptor. Thus, following such binding of agonist to receptor, the level of β-lactamase in the transfected cells increases. This increase in β-lactamase is measured by treating the cells with a cell-permeable dye that is a substrate for cleavage by β-lactamase. The dye contains two fluorescent moieties. In the intact dye, the two fluorescent moieties are close enough to one another that fluorescence resonance energy transfer (FRET) can take place between them. Following cleavage of the dye into two parts by β-lactamase, the two fluorescent moitites are located on different parts, and thus can drift apart. This increases the distance betweeen the flourescent moities, thus decreasing the amount of FRET that can occur between them. It is this decrease in FRET that is measured in the assay.

One skilled in the art can modify the assay described in Zlokarnik and U.S. Patent No. 5,741,657 to form an assay for identifying agonists of $GABA_B$ receptors by using an inducible promoter to drive β-lactamase that is activated by an

intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor. To produce the $GABA_B$ receptor, a plasmid encoding HG20 and a plasmid encoding $GABA_BR1a$ or $GABA_BR1b$ are transfected into the cells. The cells are exposed to the cell-permeable dye and then exposed to substances suspected of being agonists of the $GABA_B$ receptor. Those substances that cause a decrease in FRET are likely to actually be agonists of the $GABA_B$ receptor. To determine if the substances that are identified as agonists are sufficiently potent agonists to warrant further study, one could compare the decrease in FRET caused by the substances to the decrease in FRET that is caused by gabapentin when gabapentin is substituted for the substance in the assay.

Accordingly, the present invention includes a method for identifying agonists of the $GABA_B$ receptor comprising:

(a) providing cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of β-lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor;

(b) exposing the cells to a substrate of β-lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by β-lactamase allows the two fluorescent moietites to drift apart;

(c) measuring the amount of fluorescence resonance energy transfer (FRET) in the cells in the absence of a substance that is suspected of being an agonist of the $GABA_B$ receptor and in the absence of gabapentin;

(d) exposing the cells to the substance;

(e) measuring the amount of FRET in the cells after exposure of the cells to the substance;

(f) comparing the amount of FRET in the cells measured in step (e) to the amount of FRET measured in the cells in step (c) to obtain a value for the decrease in FRET caused by the substance;

(g) exposing the cells to gabapentin;

(h) measuring the amount of FRET in the cells after exposure of the cells to gabapentin;

(i) comparing the amount of FRET in the cells measured in step (h) to the amount of FRET measured in the cells in step (c) to obtain a value for the decrease in FRET caused by gabapentin;

comparing the decrease in FRET caused by the substance to the decrease in FRET caused by gabapentin.

By comparing the decrease in FRET caused by the substance to the decrease in FRET caused by gabapentin, one can estimate how potent an agonist the substance is. If the decrease in FRET caused by the substance is larger than the decrease in FRET caused by gabapentin, then the substance is likely to be a more potent agonist than gabapentin. If the decrease in FRET caused by the substance is about the same as the decrease in FRET caused by gabapentin, then the substance is likely to be an agonist of about the same potency as gabapentin. If the decrease in FRET caused by the substance is less than the decrease in FRET caused by gabapentin, then the substance is likely to be a weaker agonist than gabapentin.

Substeps (1)-(3) of step (a) can be practiced in any order.

The above-described assay can be modified to an assay for identifying antagonists of the $GABA_B$ receptor comprising:

(a) providing cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of β-lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of gabapentin and the $GABA_B$ receptor;

(b) exposing the cells to a substrate of β-lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by β-lactamase allows the two fluorescent moietites to drift apart;

(c) dividing the cells into a first and a second portion;

(d) exposing the first portion of the cells to gabapentin;

(e) measuring the amount of FRET in the first portion of the cells after exposure of the first portion of the cells to gabapentin;

(f) exposing the second portion of the cells concurrently to a substance that is suspected of being an antagonist of the $GABA_B$ receptor and to gabapentin;

(g) measuring the amount of FRET in the second portion of the cells after exposure of the second portion of the cells to the substance and to gabapentin;

(h) comparing the amount of FRET measured in step (e) to the amount of FRET measured in step (g);

where if the amount of FRET in step (e) is less than the amount of FRET in step (g), then the substance is an antagonist of the $GABA_B$ receptor.

Substeps (1)-(3) of step (a) can be practiced in any order.

In a particular embodiment of the above-described methods, the inducible promoter that is activated by at least one intracellular signal generated by interaction of an agonist with the $GABA_B$ receptor is a promoter that is activated by changes in membrane currents, *e.g.*, changes in potassium currents. In other embodiments, the inducible promoter is activated by the transcription factor NFAT, or is activated by a signal transduced by a chimeric Gq protein, or a signal generated by protein kinase C activity, or by changes in intracellular calcium levels.

The assays described above can be further modified to an additional assay for identifying antagonists of the $GABA_B$ receptor. Such modification would involve the use of β-lactamase under the control of a promoter that is repressed by at least one intracellular signal generated by interaction of gabapentin with the $GABA_B$ receptor and would also involve running the assay in the presence of gabapentin. When the cells are exposed to substances suspected of being antagonists of the $GABA_B$ receptor, β-lactamase will be induced, and FRET will decrease, if the substance tested is able to counteract the effect of gabapentin, *i.e.*, if the substance tested is actually an antagonist.

Accordingly, the present invention includes a method for identifying antagonists of the $GABA_B$ receptor comprising:

(a) providing cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of β-lactamase under the control of an inducible promoter that is repressed by at least one intracellular signal generated by interaction of gabapentin with the $GABA_B$ receptor;

(b) exposing the cells to gabapentin;

(c) exposing the cells to a substrate of β-lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by β-lactamase allows the two fluorescent moietites to drift apart;

(d) measuring the amount of fluorescence resonance energy transfer (FRET) in the cells in the absence of a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(e) exposing the cells to the substance;

(f) measuring the amount of FRET in the cells after exposure of the cells to the substance;

wherein if the amount of FRET in the cells measured in step (f) is less that the amount of FRET measured in the cells in step (d), then the substance is an antagonist of the $GABA_B$ receptor.

Substeps (1)-(3) of step (a) can be practiced in any order.

Steps (b) and (c) can be practiced in any order.

In a particular embodiment, the inducible promoter that is repressed by at least one intracellular signal generated by interaction of gabapentin with the $GABA_B$ receptor is a promoter that is repressed by changes in potassium currents.

In particular embodiments of the above-described methods, the cells are transfected, either stably or transiently, with expression vectors that directs the expression of HG20, $GABA_BR1a$ or $GABA_BR1b$, β-lactamase under the control of an inducible promoter that is activated by at least one intracellular signal generated by interaction of gabapentin with the $GABA_B$ receptor, and/or β-lactamase under the control of an inducible promoter that is repressed by at least one intracellular signal generated by interaction of gabapentin with the $GABA_B$ receptor.

In particular embodiments of the above-described methods, β-lactamase is TEM-1 β-lactamase from *Escherichia coli*.

In other embodiments, the subtrate of β-lactamase is CCF2/AM (Zlokarnik et al., 1998, Science 279:84-88).

In particular embodiments of the above-described methods, the inducible promoter is a promoter that is activated or repressed by NF-κB or NFAT, *e.g.*, the interleukin 2 promoter (Mattila et al., 1990, EMBO J. 9:4425-4433) or the promoter is a promoter that is regulated by cAMP levels, *e.g.*, the CRE promoter

In particular embodiments of the above-described methods, the cells express a promiscuous G-protein, *e.g.*, Gα15 or Gα16. In other embodiments, the cells have been transfected with an expression vector that directs the expression of a G-protein subunit or subunits.

The assays described above could be modified to identify inverse agonists. In such assays, one would expect a decrease in β-lactamase activity where agonists would produce an increase.

Other transcription-based assays that can be used to identify agonists and antagonists of the $GABA_B$ receptor rely on the use of reporter genes controlled by inducible promoters. Such assays can be used to identify agonists of the $GABA_B$ receptor and to determine if the agonists identified are sufficiently potent to warrant further study. This is done by providing recombinant cells that express a reporter gene under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists with the $GABA_B$ receptor. The cells are then exposed separately to gabapentin and a suspected agonist and the amount of signal from the reporter gene is measured. If the suspected agonist causes a greater signal than gabapentin, then the suspected agonist is a better agonist than gabapentin and warrants further study.

Such a method for determining if a substance is a more potent agonist of the $GABA_B$ receptor than gabapentin comprises:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of a reporter gene under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor;

(b) exposing the cells to gabapentin;

(c) measuring the amount of signal from the reporter gene in the cells that have been exposed to gabapentin;

(d) exposing the cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(e) measuring the amount of signal from the reporter gene in the cells that have been exposed to the substance;

where if the amount of signal from the reporter gene in the cells measured in step (e) is greater than the amount of signal from the reporter gene measured in the cells in step (c), then the substance is a more potent agonist of the $GABA_B$ receptor than gabapentin.

Examples of suitable reporter genes are green fluorescent protein (GFP), β-galactosidase, and luciferase. An example of such an assay for determining if a substance is a more potent agonist of the $GABA_B$ receptor than gabapentin using GFP comprises:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of green flurorescent protein (GFP) under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor;

(b) exposing the cells to gabapentin;

(c) measuring the amount of fluorescence from GFP in the cells that have been exposed to gabapentin;

(d) exposing the cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(e) measuring the amount of fluorescence from GFP in the cells that have been exposed to the substance;

where if the amount of fluorescence from GFP in the cells measured in step (e) is greater than the amount of fluorescence from GFP measured in the cells in

step (c), then the substance is a more potent agonist of the $GABA_B$ receptor than gabapentin.

Similar assays can be developed with luciferase as the reporter gene.

The above assay can be modified to an assay for identifying antagonists of the $GABA_B$ receptor. Such as assay comprises:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of green flurorescent protein (GFP) under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the $GABA_B$ receptor;

(b) exposing the cells to gabapentin;

(c) measuring the amount of fluorescence from GFP in the cells that have been exposed to gabapentin;

(d) exposing the cells to gabapentin and a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(e) measuring the amount of fluorescence from GFP in the cells that have been exposed to gabapentin and the substance;

wherein if the amount of fluorescence from GFP in the cells measured in step (e) is less than the amount of fluorescence from GFP measured in the cells in step (c), then the substance is an antagonist of the $GABA_B$ receptor.

Similar assays can be developed with luciferase or β-galactosidase as the reporter gene.

The present invention also includes assays for the identification of agonists that are more potent activators of the $GABA_B$ receptor than gabapentin and antagonists that are able to counter the effect of gabapentin on the $GABA_B$ receptor where the assays are based upon FRET between a first and a second fluorescent dye where the first dye is bound to one side of the plasma membrane of a cell expressing a heterodimer of HG20 and $GABA_BR1a$ or $GABA_BR1b$ and the second dye is free to shuttle from one face of the membrane to the other face in response to changes in membrane potential. In certain embodiments, the first dye is impenetrable to the

plasma membrane of the cells and is bound predominately to the extracellular surface of the plasma membrane. The second dye is trapped within the plasma membrane but is free to diffuse within the membrane. At normal (*i.e.*, negative) resting potentials of the membrane, the second dye is bound predominately to the inner surface of the extracellular face of the plasma membrane, thus placing the second dye in close proximity to the first dye. This close proximity allows for the generation of a large amount of FRET between the two dyes. Following membrane depolarization, the second dye moves from the extracellular face of the membrane to the intracellular face, thus increasing the distance between the dyes. This increased distance results in a decrease in FRET, with a corresponding increase in fluorescent emission derived from the first dye and a corresponding decrease in the fluorescent emission from the second dye. See figure 1 of González & Tsien, 1997, Chemistry & Biology 4:269-277. See also González & Tsien, 1995, Biophys. J. 69:1272-1280 and U.S. Patent No. 5,661,035.

In certain embodiments, the first dye is a fluorescent lectin or a fluorescent phospholipid that acts as the fluorescent donor. Examples of such a first dye are: a coumarin-labeled phosphatidylethanolamine (*e.g.*, N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); a fluorescently-labeled lectin (*e.g.*, fluorescein-labeled wheat germ agglutinin). In certain embodiments, the second dye is an oxonol that acts as the fluorescent acceptor. Examples of such a second dye are: bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol) or pentamethineoxonol analogues (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; or bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol). See González & Tsien, 1997, Chemistry & Biology 4:269-277 for methods of synthesizing various dyes suitable for use in the present invention. In certain embodiments, the assay may comprise a natural carotenoid, *e.g.*, astaxanthin, in order to reduce photodynamic damage due to singlet oxygen.

Accordingly, the present invention provides a method of identifying agonists that are more potent activators of the $GABA_B$ receptor than gabapentin comprising:

(a) providing cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of an inwardly rectifying potassium channel;

(4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and

(5) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;

(b) exposing the test cells to a substance that is suspected of being an agonist of the $GABA_B$ receptor;

(c) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells that have been exposed to the substance;

(d) exposing the test cells to gabapentin;

(e) measuring the amount of FRET in the test cells that have been exposed to gabapentin;

wherein if the amount of FRET measured in step (c) is less than the amount of FRET measured in step (e), the substance is an agonist that is a more potent activator of the $GABA_B$ receptor than gabapentin.

Of course, one of skill in the art would realize that control assays should be run where cells that lack at least one of the items recited in substeps (a) (1)-(2) are exposed to the substance and FRET is measured. The amount of FRET so measured in these control assays should be greater than the amount of FRET measured in step (c) above. This will ensure that the substance is not acting through a mechanism that has nothing to do with the $GABA_B$ receptor. In general, one of skill in the art would understand that control assays may be desirable for the assays described herein, in order to ensure that the effects measured come about through interaction of substances or gabapentin with the $GABA_B$ receptor. One of skill in the art would also understand how to set up such control assays.

The above-described assay can be modified to form a method to identify antagonists of the $GABA_B$ receptor. Such a method comprises:

(a) providing cells comprising:

(1) an expression vector that directs the expression of HG20 in the cells;

(2) an expression vector that directs the expression of $GABA_BR1a$ or $GABA_BR1b$ in the cells;

(3) an expression vector that directs the expression of an inwardly rectifying potassium channel;

(4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and

(5) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;

(b) exposing a first portion of the cells to gabpentin in the presence of a substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(c) exposing a second portion of the cells to gabpentin in the absence of the substance that is suspected of being an antagonist of the $GABA_B$ receptor;

(d) measuring the amount of fluorescence resonance energy transfer (FRET) in the cells of steps (b) and (c);

(e) comparing the amount of FRET measured in the cells of steps (b) and (c);

where if the amount of FRET measured in the cells of step (b) is greater than the amount of FRET measured in the cells of step (c), the substance is an antagonist of the $GABA_B$ receptor.

Inwardly rectifying potassium channels that are suitable for use in the methods of the present invention are disclosed in, *e.g.*, Misgeld et al., 1995, Prog. Neurobiol. 46:423-462; North, 1989, Br. J. Pharmacol. 98:13-23; Gahwiler et al.,1985, Proc. Natl. Acad. Sci USA 82:1558-1562; Andrade et al., 1986, Science 234:1261.

In particular embodiments of the above-described methods, the first fluorescent dye is selected from the group consisting of: a fluorescent lectin; a fluorescent phospholipid; a coumarin-labeled phosphatidylethanolamine; N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine); N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-

dipalmitoylphosphatidylethanolamine); and fluorescein-labeled wheat germ agglutinin.

In particular embodiments of the above-described methods, the second fluorescent dye is selected from the group consisting of: an oxonol that acts as the fluorescent acceptor; bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols; bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol; bis(1,3-dialkyl-2-thiobarbiturate) quatramethineoxonols; bis(1,3-dialkyl-2-thiobarbiturate)pentamethineoxonols; bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol); and bis(1,3-dialkyl-2-thiobarbiturate) hexamethineoxonols.

The $GABA_B$ receptor belongs to the class of proteins known as G-protein coupled receptors (GPCRs). GPCRs transmit signals across cell membranes upon the binding of ligand. The ligand-bound GPCR interacts with a heterotrimeric G-protein, causing the Gα subunit of the G-protein to disassociate from the Gβ and G γ subunits. The Gα subunit can then go on to activate a variety of second messenger systems.

Generally, a particular GPCR is only coupled to a particular type of G-protein. Thus, to observe a functional response from the GPCR, it is necessary to ensure that the proper G-protein is present in the system containing the GPCR. It has been found, however, that there are certain G-proteins that are "promiscuous." These promiscuous G-proteins will couple to, and thus transduce a functional signal from, virtually any GPCR. See Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 (Offermanns). Offermanns described a system in which cells are transfected with expression vectors that result in the expression of one of a large number of GPCRs as well as the expression of one of the promiscuous G-proteins Gα15 or Gα 16. Upon the addition of an agonist of the GPCR to the transfected cells, the GPCR was activated and was able, via Gα15 or Gα16, to activate the β isoform of phospholipase C, leading to an increase in inositol phosphate levels in the cells. In addition to the G-protein described by Offermans, chimeric G-proteins, such as Gqi5, also exhibit promiscuous coupling of GPCRs to the phospholipase C pathway. Therefore, the present invention includes assays that are essentially the same as the assays described herein using promiscuous G-proteins except that chimeric G-proteins are used instead of promiscuous G-proteins. Chimeric G-proteins are described in, *e.g.*, Joshi et al, 1999, Eur. J. Neurosci. 11:383-388.

Therefore, by making use of these promiscuous G-proteins, it is possible to set up functional assays for the $GABA_B$ receptor, even in the absence of knowledge of the G-protein with which the $GABA_B$ receptor is coupled *in vivo*. One possibility for utilizing promiscuous G-proteins in connection with the $GABA_B$ receptor includes a method of identifying an agonist that is a more potent activator of the $GABA_B$ receptor than gabapentin comprising:

(a) providing cells that express HG20, $GABA_BR1a$ or $GABA_BR1b$, and a promiscuous G-protein, where HG20 and either $GABA_BR1a$ or $GABA_BR1b$ form a heterodimer representing a functional $GABA_B$ receptor;

(b) exposing a first portion of the cells to a substance that is a suspected agonist of the $GABA_B$ receptor;

(c) measuring the level of inositol phosphates in the cells that have been exposed to the substance;

(d) exposing a second portion of the cells to gabapentin;

(e) measuring the level of inositol phosphates in the cells that have been exposed to gabapentin;

where if the level of inositol phosphates measued in step (c) is greater than the level of inositol phosphates measured in step (e), then the substance is a more potent activator of the $GABA_B$ receptor than gabapentin.

Levels of inositol phosphates can be measured by monitoring calcium mobilization. Intracellular calcium mobilization is typically assayed in whole cells under a microscope using fluorescent dyes or in cell suspensioins via luminescence using the aequorin assay.

In methods related to those described above, rather than using changes in inositol phosphate levels as an indication of $GABA_B$ receptor function, potassium currents are measured. This is feasible since the $GABA_B$ receptor, like other metabotropic receptors, is coupled to potassium channels. Thus, one could measure $GABA_B$ receptor coupling to GIRK1, GIRK2, GIRK3, GIRK4, or to other potassium channels in oocytes. GIRKs, methods of manipulating oocytes, and methods of measuring potassium channel activity in oocytes and HEK 293 cells are described in Goldin, 1992, Meth. Enzymol. 207:266-279; Quick & Lester, 1994, Meth. Neurosci. 19:261-279; Smith et al., 1998, J. Cell Biol. 273:23321-23326; Kubo et al., 1997, Nature 364:802-806; Krapivinsky et al., 1995, Nature 374:135-141; Dascal et al., 1993, Proc. Natl. Acad. Sci. USA 90:10235-10239; Jones et al., 1998, Nature

396:674-679; White et al., 1998, Nature 396:679-682; Kaupmann et al., 1998, Nature 396:683-687; Kuner et al., 1999, Science 283:74-77; and references cited therein.

In a particular embodiment of the above-described method, the promiscuous G-protein is selected from the group consisting of Gα15, Gα16, and chimeric G-proteins such as Gqi5. Expression vectors containing Gα15 or Gα16 are known in the art. See, *e.g.*, Offermanns; Buhl et al., 1993, FEBS Lett. 323:132-134; Amatruda et al., 1993, J. Biol. Chem. 268:10139-10144.

The above-described assay can be modified to form a method to identify antagonists of the $GABA_B$ receptor. Such a method is also part of the present invention and comprises:

(a) providing cells that express HG20, $GABA_BR1a$ or $GABA_BR1b$, and a promiscuous G-protein;

(b) exposing the cells to gabpentin in the presence and in the absence of a substance that is a suspected antagonist of the $GABA_B$ receptor;

(c) measuring the level of inositol phosphates in the cells;

where a decrease in the level of inositol phosphates in the cells in the presence of the suspected antagonist as compared to the level of inositol phosphates in the cells in the absence of the suspected antagonist indicates that the substance is an antagonist of the $GABA_B$ receptor.

In particular embodiments of the above-described methods, the cells are transfected with expression vectors that direct the expression of HG20, $GABA_BR1a$ or $GABA_BR1b$, inwardly rectifying potassium channels, and/or the promiscuous G-protein in the cells. The cells are then cultured under conditions such that HG20, $GABA_BR1a$ or $GABA_BR1b$, inwardly rectifying potassium channels, and/or the promiscuous G-protein are expressed and functional $GABA_B$ receptors are formed. The assays described above generally can be carried out with cells that have been transiently or stably transfected. Transfection is meant to include any method known in the art for introducing expression vectors into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct, and electroporation. In particular embodiments, a single expression vector encodes HG20 and $GABA_BR1a$ or $GABA_BR1b$.

In a particular embodiments of the above-described methods, the promiscuous G-protein is selected from the group consisting of Gα15, Gα16, and chimeric G-proteins such as Gqi5.

For convenience of detection in the above-described methods, gabapentin or the substances may be labeled, *e.g.*, radioactively, enzymatically, fluorescently, *etc.*

Once a substance has been identified by the above-described methods, determining whether the substance is an agonist or antagonist can then be accomplished by the use of functional assays such as those described herein.

In particular embodiments, the binding affinity of the substance for heterodimers of HG20 and $GABA_BR1a$ or $GABA_BR1b$ is determined. In particular embodiments, such binding affinity is between 1nM and 200 mM; preferably between 5 nM and 1 mM; more preferably between 10 nM and 100 μM; and even more preferably between 10 nM and 100 nM.

The conditions under which cells are exposed to gabapentin or substances in the above-described method are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments, the assays described above are run in the presence of an allosteric modulator of the $GABA_B$ receptor, *i.e.*, a compound that binds proximal to the agonist binding site to increase or decrease the affinity of the agonist. The assays described above can be modified to become assays for identifying allosteric modulators of the $GABA_B$ receptor by running the assays in the presence of the agonist gabapentin and exposing the assay cells to substances that are suspected of being allosteric modulators. Those substances that bind proximal to the gabapentin binding site to increase or decrease the affinity of gabapentin are actually allosteric modulators.

In a particular embodiments of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2),

C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* oocytes, or *Xenopus* melanophores.

In particular embodiments of the above-described methods, HG20 is a polypeptide comprising an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;
Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2;
Positions 57-941 of SEQ.ID.NO.:2;
the amino acid sequence encoded by the DNA sequence deposited in GenBank accession no. AF056085;
the amino acid sequence encoded by the DNA sequence deposited in GenBank accession no. AJ012188; and
the amino acid sequence encoded by the DNA sequence deposited in GenBank accession no. ASF074482.

In particular embodiments of the above-described methods, HG20 is a chimeric HG20 protein. By chimeric HG20 protein is meant a contiguous polypeptide sequence of HG20 fused in frame to a polypeptide sequence of a non-HG20 protein. For example, the N-terminal domain and seven transmembrane spanning domains of HG20 fused at the C-terminus in frame to a G protein is a chimeric HG20 protein. Another example of a chimeric HG20 protein is a polypeptide comprising the FLAG epitope fused in frame at the amino terminus of amino acids 52-941 of SEQ.ID.NO.:2. Especially preferred forms of chimeric HG20 proteins are those in which a non-HG20 polypeptide replaces a portion of the N-terminus of HG20.

Chimeric $GABA_BR1a$ and $GABA_BR1b$ proteins may also be used in the present invention. In particular embodiments, the chimeric $GABA_BR1a$ or

GABA$_B$R1b protein comprises the entire coding region of GABA$_B$R1a or GABA$_B$R1b except for the signal sequence fused in frame to a polypeptide sequence of a non-GABA$_B$R1a or GABA$_B$R1b protein.

In particular embodiments, the expression vector encoding HG20 comprises a nucleotide sequence selected from the group consisting of:

Positions 293-3,115 of SEQ.ID.NO.:1;
Positions 317-3,115 of SEQ.ID.NO.:1;
Positions 395-3,115 of SEQ.ID.NO.:1;
Positions 398-3,115 of SEQ.ID.NO.:1;
Positions 404-3,115 of SEQ.ID.NO.:1;
Positions 407-3,115 of SEQ.ID.NO.:1;
Positions 416-3,115 of SEQ.ID.NO.:1;
Positions 422-3,115 of SEQ.ID.NO.:1;
Positions 428-3,115 of SEQ.ID.NO.:1;
Positions 446-3,115 of SEQ.ID.NO.:1; and
Positions 461-3,115 of SEQ.ID.NO.:1.

In particular embodiments of the above-described methods, GABA$_B$R1a is a polypeptide comprising an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:21;
the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246;:239-246;
SEQ.ID.NO.:22; and
the protein encoded by SEQ.ID.NO.:24.

In particular embodiments of the above-described methods, GABA$_B$R1b is rat GABA$_B$R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386: 239-246 or is human GABA$_B$R1b and has the amino acid sequence encoded by the DNA sequence deposited in GenBank accession no. AJ012186.

The assays descibed above could be modified to identify inverse agonists. In such assays, inverse agonists would be identified through a change in the signal that is being assayed that is the opposite of the change that is observed with an agonist. For example, in the assays using β-lactamase reporter genes, inverse agonists would lead to a decrease in β-lactamase activity under conditions where agonists lead

to an increase. Similarly, inverse agonists can be identified by modifying the functional assays that were described previously where those functional assays monitored decreases in cAMP levels. In the case of assays for inverse agonists, increases in cAMP levels would be observed.

Some of the above-described methods can be modified in that, rather than exposing cells to the substance or to gabapentin, membranes can be prepared from the cells and those membranes can be exposed to the substance or gabapentin. Such a modification utilizing membranes rather than cells is well known in the art with respect to assays involving other receptors and is described in, *e.g.*, Hess et al., 1992, Biochem. Biophys. Res. Comm. 184:260-268.

Some of the above-described methods can be modified to take advantage of other ways of assaying for agonist activity at the $GABA_B$ receptor. Agonists/antagonists/inverse agonists may affect the internalization or trafficking of functional $GABA_B$ receptors. For example, in the case of the β2-adrenergic receptor, agonist exposure results in receptor internalization. Therefore, receptor trafficking between intracellular pools and the cytoplasmic membrane may be considered an assay of agonist activity. It may be that $GABA_B$ receptor trafficking is modulated by agonists in a similar manner. It would then be possible to identify agonist activity by monitoring $GABA_B$ receptor trafficking. Such trafficking can be monitored by whole cell immunohistochemistry and confocal microscopy or by surface and intracellular receptor labeling and flow cytometry.

Furthermore, because the functional $GABA_B$ receptor may be a heterodimer, then agonists/antagonists/inverse agonists may be expected to alter the ratio of heterodimer to monomer. Hence the disruption or appearance of a heterodimer may be considered an additional screening assay. In this case, the monitoring of receptor dimerization or disappearance may be done by the appearance or disruption of FRET. Each of the monomers are labelled with a fluorophore such that close proximity would allow FRET to occur. Upon agonist binding, one might see disruption of FRET, indicating disruption of dimers or increase in FRET indicating more dimerization in the course of agonist activation.

Another possibility is to use a microphysiometer to monitor agonist activity. The activation of many receptor pathways is associated with changes in extracellular or intracellular pH. Thus, $GABA_B$ receptor agonists can likely be identified by the use of a microphysiometer to detect such changes when cells

expressing $GABA_B$ receptors are exposed to suspected agonists. The use of microphysiometers is described in Ng et al., 1999, J. Cell. Biochem. 72:517-527 and Fischer et al., 1999, J. Membr. Biol. 168:39-45.

While the above-described methods are explicitly directed to testing whether "a" substance is an agonist or antagonist of the $GABA_B$ receptor, it will be clear to one skilled in the art that such methods can be adapted to test collections of substances, *e.g.*, combinatorial libraries, collections of natural produces, *etc.*, to determine whether any members of such collections are activators or inhibitors of the $GABA_B$ receptor. Accordingly, the use of collections of substances, or individual members of such collections, as the substance in the above-described methods is within the scope of the present invention.

The present inventors have discovered a pharmacological difference between the $GABA_B$ receptor agonists GABA on the one hand and gabapentin and baclofen on the other. GABA is able to activate a functional response of the $GABA_B$ receptor when that functional response is measured either by monitoring changes in inwardly rectifying potassium currents in *Xenopus* oocytes or by monitoring pigment aggregation in *Xenopus* melanophores. In contrast, while gabapentin and baclofen are capable of activating a functional response of the $GABA_B$ receptor when that functional response is measured by monitoring changes in inwardly rectifying potassium currents in *Xenopus* oocytes, gabapentin and baclofen are not capable of activating a functional response of the $GABA_B$ receptor when that functional response is measured by monitoring pigment aggregation in *Xenopus* melanophores.

Figure 10A-F illustrates this. Figure 10A shows that GABA mediates pigment aggregation in oocytes that co-express FLAG-HG20 and murine $GABA_BR1a$ (with an EC_{50} of 7.7 μM in this series of experiments). In contrast, baclofen is inactive at similar concentrations, only showing non-specific activity at roughly millimolar concentrations (Figure 10B). Note that even oocytes expressing a control vector, pcDNA3.1, and not expressing either HG20 or $GABA_BR1a$, also show some activity at millimolar baclofen concentrations. This demonstrates that the activity of baclofen at such concentrations is not specific to the expression of HG20/$GABA_BR1a$ heterodimers. Figure 10C shows that the effect of GABA is abolished by co-treatment with the $GABA_B$ antagonist CGP71872. Figure 10D shows that CGP71872 has no effect on the results seen with baclofen. Figure 10E shows that the effect of GABA is not mediated by metabotropic glutamate receptor 4

or by dimers of metabotropic glutamate receptor 4 and either HG20 or GABA$_B$R1a. Figure 10F shows that, like baclofen, gabapentin is unable to cause pigment aggregation in oocytes.

The present inventors believe that the results described above point to the existence of a subset of GABA$_B$ receptor agonists that have an unexpected pharmacological activity. These agonists can couple the GABA$_B$ receptor to ion channels but do not couple the GABA$_B$ receptor to changes in pigment aggregation in *Xenopus* melanophores. Gabapentin and baclofen are examples of such agonists. Thus, as Figure 10A-F shows, gabapentin and baclofen are able to modulate inwardly rectifying potassium currents in *Xenopus* oocytes, but are unable to cause pigment aggregation in *Xenopus* melanophores. The *Xenopus* melanophore pigment aggregation/dispersion assay has been shown to be highly suitable for monitoring agonist activation of Gi-, Gq-, and Gs-coupled receptors (Potenza et al., 1992, Anal. Biochem. 206:315-322; Lerner, 1994, Trends Neurosci. 17:142-146). Agonist activation of Gi-coupled receptors expressed in melanophores results in pigment aggregation via a reduction in intracellular cAMP levels, whereas activation of Gs- and Gq-coupled receptors results in pigment dispersion via elevations in intracellular cAMP and calcium levels, respectively. The inability of gabapentin or baclofen to cause pigment aggregation in this assay argues that these agonists are incapable of coupling GABA$_B$ receptors to changes in pigment aggregation in *Xenopus* melanophores.

These observations allow for the development of assays to identify substances that are gabapentin-like agonists of the GABA$_B$ receptor. By gabapentin-like agonist is meant a substance that, like gabapentin and baclofen, is able to to couple the activity of the GABA$_B$ receptor to ion channels but is not able to couple the activity of the GABA$_B$ receptor to changes in pigment aggregtion in melanophores. Given the proven pharmacological utility of gabapentin and baclofen, it would be highly desirable to identify more such gabapentin-like agonists. Accordingly, the present invention includes a method of identifying substances that are gabapentin-like agonists of the GABA$_B$ receptor where the method comprises:

(a) determining whether a substance is able to to couple the activity of the GABA$_B$ receptor to ion channels;

(b) determining whether a substance is able to couple the activity of the GABA$_B$ receptor to changes in pigment aggregation in *Xenopus* melanophores;

where if the substance is is able to to couple the activity of the $GABA_B$ receptor to ion channels but is not able to couple the activity of the $GABA_B$ receptor to changes in pigment aggregation in *Xenopus* melanophores, then the substance is a gabapentin-like agonist of the $GABA_B$ receptor.

Methods disclosed herein can be used to determine whether a substance is able to to couple the activity of the $GABA_B$ receptor to ion channels or whether a substance is able to couple the activity of the $GABA_B$ receptor to changes in pigment aggregation in *Xenopus* melanophores. Of course, other methods well known in the art can also be used.

In specific embodiments, step (a) of the method comprises injecting *Xenopus* oocytes with RNA encoding HG20, a $GABA_BR1a$ or $GABA_BR1b$ subunit, and at least one GIRK potassium channel and measuring changes in inwardly rectifying potassium currents when the oocytes are exposed to the substance. In specific embodiments, the GIRK potassium channel is selected from the group consisting of: GIRK1, GIRK2, GIRK3, and GIRK4.

In specific embodiments, step (a) of the method comprises monitoring changes in FRET between a first and a second fluorescent dye where the first dye is bound to one side of the plasma membrane of a cell expressing a heterodimer of HG20 and $GABA_BR1a$ or $GABA_BR1b$ and the second dye is free to shuttle from one face of the membrane to the other face in response to changes in membrane potential.

In specific embodiments, step (a) of the method comprises measuring changes in K^+ or Ca^{++} currents. In specific embodiments, the changes are increases in K^+ or decreases in Ca^{++} currents.

In particular embodiments of the above-described methods, the substance is a gabpentin-like compound that is produced by modifying the structure of gabapentin by methods of medicinal chemistry. As is well known in the art, it is common to modify a "lead" compound having a particular pharmacological activity (*e.g.*, gabapentin) by sequentially replacing the functional groups of the compound (*e.g.*, amine groups, methyl groups, carboxyl groups, phenolic groups, azido groups, *etc.*) with different functional groups and testing the modified compounds to determne what effect such replacement has on the compound's pharmacological properties. In such a manner, compounds having improved pharmacological properties such as higher target specificity, more potent agonist or antagonist activity, or lower toxicity can be developed. Comparison of the structures of such modified compounds with

the pharmacological properties of the modified compounds can be especially informative in suggesting portions of the compounds which should be conserved and portions which should be varied in order to arrive at a compound with optimal properties. Methods of medicinal chemistry such as these can be applied to gabapentin and the modified gabapentin-like compounds so produced can be tested in the various methods described herein to determine if they possess desirable properties such as, *e.g.*, the property of being a gabapentin-like agonist.

The modified gabapentin-like compounds can also be tested by the assays described herein to determine if they are agonists or antagonists of the $GABA_BR1b$ receptor. By comparing the functional responses of the gabapentin-like compounds to the responses of gabapentin, one can determine if the gabapentin-like compounds are more potent agonists than gabapentin.

Agonists and antagonists identified by the above-described methods are useful in the same manner as well-known agonists and antagonists of $GABA_B$ receptors. For example, (-)baclofen is a known agonist of $GABA_B$ receptors and, in racemic form, is a clinically useful muscle relaxant known as LIORESAL® (Bowery & Pratt, 1992, Arzneim.-Forsch./Drug Res. 42:215-223 [Bowery & Pratt]). Gabapentin has been sold since 1994 in the United States as a treatment for epilepsy under the name NEURONTIN®. Bowery & Pratt, at Table 1, page 219, list the therapeutic potential of $GABA_B$ receptor agonists and antagonists. For agonists, the therapeutic potential is said to include use as muscle relaxants and anti-asthmatics. For antagonists, the therapeutic potential is said to include use as antidepressants, anticonvulsants, nootropics, and anxiolytics. Additionally, at page 220, left column, Bowery & Pratt list some additional therapeutic uses for the $GABA_B$ receptor agonist (-)baclofen: treatment of trigeminal neuralgia and reversal of ethanol withdrawal symptoms. Given the wide range of utility displayed by known agonists and antagonists of $GABA_B$ receptors, it is clear that those skilled in the art would consider the agonists and antagonists identified by the methods of the present invention to be pharamacologically useful. In addition, it is believed that such agonists and antagonists will also be useful in the treatment of epilepsy, neuropsychiatric disorders, and dementias.

Methods of making gabapentin and gabapentin-like compounds are described in U.S. Patent No. 4,024,175 and U.S. Patent No. 4,152,326.

The following non-limiting examples are presented to better illustrate the invention.

EXAMPLE 1

Cloning of HG20

A cDNA fragment encoding full-length HG20 can be isolated from a human fetal brain cDNA library by using the polymerase chain reaction (PCR) employing the following primer pair:

HG20.F139	5'-CCGTTCTGAGCCGAGCCG -3' (SEQ.ID.NO.:3)
HG20.R3195	5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

The above primer pair is meant to be illustrative only. Those skilled in the art would recognize that a large number of primer pairs, based upon SEQ.ID.NO.:1, could also be used.

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq Gold, Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM $MgCl_2$, 200 μM for each dNTP, 50 mM KCl, 0.2 μM for each primer, 10 ng of DNA template, 0.05 units/μl of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using the cycling parameters of 95°C, 20 seconds, 62°C, 20 seconds, 72°C, 3 minutes. In addition to these conditions, a variety of suitable PCR protocols can be found in PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press.

A suitable cDNA library from which a clone encoding HG20 can be isolated would be a random primed fetal brain cDNA library consisting of approximately 4.0 million primary clones constructed in the plasmid vector pBluescript (Stratagene, LaJolla, CA). The primary clones of such a library can be subdivided into pools with each pool containing approximately 20,000 clones and each pool can be amplified separately.

By this method, a cDNA fragment (SEQ.ID.NO.:1) encoding an open reading frame of 941 amino acids (SEQ.ID.NO.:2) is obtained. This cDNA fragment

can be cloned into a suitable cloning vector or expression vector. For example, the fragment can be cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA). HG20 protein can then be produced by transferring an expression vector containing SEQ.ID.NO.:1 or portions thereof into a suitable host cell and growing the host cell under appropriate conditions. HG20 protein can then be isolated by methods well known in the art.

Alternatively, other cDNA libraries made from human tissues that express HG20 RNA can be used with PCR primers HG20.F139 and HG20.R3195 in order to amplify a cDNA fragment encoding full-length HG20. Suitable cDNA libraries would be those prepared from cortex, cerebellum, testis, ovary, adrenal gland, thyroid, or spinal cord.

As an alternative to the above-described PCR method, a cDNA clone encoding HG20 can be isolated from a cDNA library using as a probe oligonucleotides specific for HG20 and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described in, *e.g.*, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K., Vol. I, II. Oligonucleotides that are specific for HG20 and that can be used to screen cDNA libraries are:

HG20.F46	5'-GGGATGATCATGGCCAGTGC-3' (SEQ.ID.NO.:5)
HG20.R179	5'-GGATCCATCAAGGCCAAAGA-3' (SEQ.ID.NO.:6)
HG21.F43	5'-GCCGCTGTCTCCTTCCTGA-3' (SEQ.ID.NO.:7)
HG21.R251	5'-TTGGTTCACACTGGTGACCGA-3' (SEQ.ID.NO.:8)
HG20.R123	5'-TTCACCTCCCTGCTGTCTTG-3' (SEQ.ID.NO.:9)
HG20.F1100	5'-CAGGCGATTCCAGTTCACTCA-5' (SEQ.ID.NO.:10)
HG20.F1747	5'-GAACCAAGCCAGCACATCCC-3' (SEQ.ID.NO.:11)
HG20.R54	5'-CCTCGCCATACAGAACTCC-3' (SEQ.ID.NO.:12)
HG20.R75	5'-GTGTCATAGAGCCGCAGGTC-3' (SEQ.ID.NO.:13)
HG20.F139	5'-CCGTTCTGAGCCGAGCCG-3' (SEQ.ID.NO.:3)
HG20.R3195	5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

Membrane-spanning proteins, such as $GABA_B$ receptor subunits, when first translated generally possess an approximately 16 to 40 amino acid segment known as a signal sequence. Signal sequences direct the nascent protein to be transported through the endoplasmic reticulum membrane, following which signal sequences are cleaved from the protein. Signal sequences generally contain from 4 to 12 hydrophobic residues but otherwise possess little sequence homology. The Protein Analysis tool of the GCG program (Genetics Computer Group, Madison, Wisconsin), a computer program capable of identifying likely signal sequences, was used to examine the N terminus of HG20. Several likely candidates for cleavage sites which would generate mature HG20 protein, *i.e.*, protein lacking the signal sequence, were identified. In view of these likely signal sequences, the assays of the present invention can be practiced with HG20 proteins having the following sequences:

Positions 9-941 of SEQ.ID.NO.:2;
Positions 35-941 of SEQ.ID.NO.:2;
Positions 36-941 of SEQ.ID.NO.:2;
Positions 38-941 of SEQ.ID.NO.:2;
Positions 39-941 of SEQ.ID.NO.:2;
Positions 42-941 of SEQ.ID.NO.:2;
Positions 44-941 of SEQ.ID.NO.:2;
Positions 46-941 of SEQ.ID.NO.:2;
Positions 52-941 of SEQ.ID.NO.:2;
Positions 57-941 of SEQ.ID.NO.:2.

EXAMPLE 2

Construction of Full Length Murine $GABA_BR1a$ Coding Region

Using a combination of TFASTX (Pearson et al., 1997, Genomics 46:24-36) and TBLASTX (Altschul et al., 1997, Nucleic Acids Res. 25:3389-3402) searching programs against dbEST: Database of Expressed Sequence Tags (URL http://www.ncbi.nlm.nih.gov/dbEST/index.html), we identified partial cDNA clones in the EST collection which encoded murine $GABA_BR1a$ using the rat $GABA_BR1a$ subunit cDNAs (GenBank Accession Numbers Y10369 and Y10370) as probe sequences (Kaupmann et al., 1997, Nature

386:239-246). Two of these ESTs (IMAGE Consortium clone identification numbers 472408 and 319196) were obtained (Research Genetics, Birmingham, Ala). The DNA sequences of both cDNA clones were determined using standard methods on an ABI 373a automated sequencer (Perkin-Elmer-Applied Biosystems, Foster City, CA).

The partial cDNAs were assembled by long accurate PCR using the following oligonucleotides: 472408 sense: 5' - GC GAATTC GGTACC ATG CTG CTG CTG CTG CTG GTG CCT - 3' (SEQ.ID.NO.:14), 472408 antisense: 5' – GG GAATTC TGG ATA TAA CGA GCG TGG GAG TTG TAG ATG TTA AA - 3' (SEQ.ID.NO.:15), 319196 sense: 5' - CCA GAATTC CCA GCC CAA CCT GAA CAA TC - 3' (SEQ.ID.NO.:16), 319196 antisense: 5' - CG GCGGCCGC TCA CTT GTA AAG CAA ATG TA - 3' (SEQ.ID.NO.:17) which amplified two fragments corresponding to the 5' 2,100 basepairs and 3' 1,000 basepairs of the murine $GABA_BR1a$ coding region. The PCR conditions were 200 ng of cDNA template, 2.5 units of Takara LA Taq (PanVera, Madison, WI), 25 mM TAPS (pH 9.3), 50 mM KCl, 2.5 nM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 mM each dNTP and 1 mM each primer with cycling as follows 94°C 1 min, 9 cycles of 98°C for 20 seconds, 72°C-56 °C (decreases 2°C per cycle), 72°C for 30 seconds, followed by 30 cycles of 98°C for 20 seconds, 60°C for 3 minutes. A final extension at 72°C for 10 minutes was performed. PCR products were cloned into the TA-Cloning vector pCRII-TOPO (Invitrogen, San Diego, CA) following the manufacturers directions. Cloned PCR products were confirmed by DNA sequencing. To form full-length cDNA, the pCINeo mammalian expression vector was digested with EcoRI and NotI. The EcoRI fragment from PCR cloning of 472408 and the EcoRI/NotI product from PCR cloning of 319196 were ligated in a three part ligation with digested pCINeo vector. The resulting clones were screened by restriction digestion with SstI which cuts once in the vector and once in the 472408 derived fragment. The resulting expression clone is 2,903 basepairs in length. The overall cDNA length, including untranslated sequences, inferred from the full length of the two ESTs is 4,460 basepairs. Expression vectors encoding $GABA_BR1a$ were prepared by subcloning full-length $GABA_BR1a$ into the NheI-NotI site of pcDNA3.1 or pCIneo.

EXAMPLE 3

Construction of the FLAG epitope-tagged HG20

The FLAG epitope-tagged HG20 receptor subunit was constructed by PCR using a sense primer encoding a modified influenza hemaglutinin signal sequence (MKTIIALSYIFCLVFA; SEQ.ID.NO.:25) (Jou et al., 1980, Cell 19:683-696) followed by an antigenic FLAG epitope (DYKDDDDK; SEQ.ID.NO.:26) and DNA encoding amino acids 52-63 of HG20 and an antisense primer encoding amino acids 930-941 of the HG20 in a high-fidelity PCR reaction with HG20/pCR 3.1 as a template. HG20/pCR 3.1 is a plasmid that contains full-length HG20 (SEQ.ID.NO.:2) cloned into pCR3.1. The nucleotide sequences of the sense and antisense primers are: sense: 5'-GCC GCT AGC GCC ACC ATG AAG ACG ATC ATC GCC CTG AGC TAC ATC TTC TGC CTG GTA TTC GCC GAC TAC AAG GAC GAT GAT GAC AAG AGC AGC CCG CCG CTC TCC ATC ATG GGC CTC ATG CCG CTC-3', (SEQ.ID.NO.:18); antisense: 5'-GCC TCT AGA TTA CAG GCC CGA GAC CAT GAC TCG GAA GGA GGG TGG CAC-3'. (SEQ.ID.NO.:19). The PCR conditions were: precycle denaturation at 94°C for 1 min, 94°C for 30 sec, annealing and extension at 72°C for 4 min for 25 cycles, followed by a 7 min extension at 72°C . The PCR product, SF-HG20 DNA, flanked by NheI and XbaI sites was subcloned into the NheI/XbaI site of pcDNA3.1 (Invitrogen, San Diego, Ca) to give rise to the expression construct SF-HG20/pcDNA3.1. The sequence of this construct was verified on both strands.

EXAMPLE 4

Kir channel activity in *Xenopus* oocytes

With the following modifications, *Xenopus* oocytes were isolated as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261) from live frogs supplied by Boreal, Inc. After a brief (10 min) hypertonic shock with 125 mM potassium phosphate pH 6.5, oocytes were allowed to recover in Barth's solution for 1-2 hr. cDNA constructs for human Kir 3.1, Kir 3.2 channel isoforms (generous gifts from Dr. Hubert Van Tol, University of Toronto), and Giα1 (a generous gift of Dr.

Maureen Linder, Washington University) were linearized by restriction enzymes and purified using Geneclean (Bio 101). Murine $GABA_BR1a$ or FLAG-HG20 clones were subcloned into pT7TS (a generous gift of Dr. Paul Krieg, University of Texas) before linearization and transcription. Capped cRNA was made using T7 RNA polymerase and the mMessage mMachine (Ambion). Individual oocytes were injected with 5-10 ng (in 25-50 nL) of Kir3.1 and Kir3.2 constructs with mRNAs for murine $GABA_BR1a$ or FLAG-HG20 and in combination with $Gi\alpha 1$ as well. Kir currents were also evaluated in ooctyes co-injected with Kir3.1, Kir3.2, murine $GABA_BR1a$ and FLAG-HG20 mRNAs. Currents were recorded after 48 hr. Standard recording solution was KD-98, 98 mM KCl, 1 mM $MgCl_2$, 5 mM K-HEPES, pH 7.5, unless otherwise stated. Microelectrodes were filled with 3 M KCl and had resistances of 1-3 MW and 0.1-0.5 MW for voltage and current electrodes, respectively. In addition, current electrodes were backfilled with 1% agarose (in 3M KCl) to prevent leakage as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261). Recordings were made at room temperature using a Geneclamp 500 amplifier (Axon Instruments). Oocytes were voltage clamped and perfused continuously with different recording solutions. Currents were evoked by 500 msec voltage commands from a holding potential of -10 mV, delivered in 20 mV increments from -140 to 60 mV to test for inward rectifying potassium currents. Data were recorded at a holding potential of -80 mV and drugs were added to the bath with a fast perfusion system. Data collection and analysis were performed using pCLAMP v6.0 (Axon Instruments) and Origin v4.0 (MicroCal) software. For subtraction of endogenous and leak currents, records were obtained in ND-96, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Na-HEPES and these were subtracted from recordings in KD-98 before further analysis.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.